MOLECULAR AND CELLULAR BIOLOGY, Oct. 1990, p. 5305–5313
0270-7306/90/105305-09$02.00/0

Vol. 10, No. 10

Cross-Linking of T-Cell Surface Molecules CD4 and CD8 Stimulates Phosphorylation of the *lck* Tyrosine Protein Kinase at the Autophosphorylation Site

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p56^{lck}, a lymphocyte-specific tyrosine protein kinase, binds to the cytoplasmic tails of the T-cell surface molecules CD4 and CD8. Cross-linking of CD4 expressed on the surface of murine thymocytes, splenocytes, and CD4$^+$ T-cell lines induced tyrosine phosphorylation of p56^{lck} dramatically. Cross-linking of CD8 stimulated tyrosine phosphorylation of p56^{lck} strongly in murine L3 and GA4 cells, slightly in splenocytes, but not detectably in thymocytes. Differing effects of cross-linking on in vitro tyrosine kinase activity of p56^{lck} were observed. An increase in the in vitro kinase activity of p56^{lck}, when assayed with [Val^5]-angiotensin II as an exogenous substrate, was found to accompany cross-linking of CD4 in three cell lines. No stimulation of the in vitro kinase activity, however, was observed after cross-linking of CD8 in L3 cells. The phosphorylation of p56^{lck} at Tyr-394, the autophosphorylation site, was stimulated by cross-linking in all cell lines examined. Tyr-394 was the predominant site of increased tyrosine phosphorylation in two leukemic cell lines. In the other two cell lines, the phosphorylation of both Tyr-394 and an inhibitory site, Tyr-505, was found to increase. In contrast to cross-linking with antibodies, no striking increase in the tyrosine phosphorylation of p56^{lck} was stimulated by antigenic stimulation. Therefore, the effect of antibody-induced aggregation of CD4 and CD8 on the tyrosine phosphorylation of p56^{lck} differs, at least quantitatively, from what occurs during antigen-induced T-cell activation.

lck is a member of the *src* family of genes that encode cytoplasmic protein tyrosine kinases. Its product, p56^{lck}, is expressed almost exclusively in lymphoid cells and tissues (6, 22, 28, 41, 47). Like other protein tyrosine kinases, p56^{lck} is thought to play a role in the generation of growth regulatory signals. p56^{lck} binds to the cytoplasmic tails of the T-cell surface molecules CD4 and CD8 in both human and murine T cells (35, 38, 43, 50). Its association with CD4 and CD8 suggests that it may play a role in a signal transduction pathway during T-cell activation or T-cell maturation.

CD4 and CD8 bind to class II and class I major histocompatibility proteins, respectively, during antigen-induced T-cell activation (8, 11, 30–32) and during T-cell maturation (9, 21, 37). In addition to increasing adhesion between T cells and antigen-presenting cells, several lines of evidence suggest the involvement of CD4 and CD8 in transducing growth regulatory signals. It has been demonstrated that a truncated form of CD4 that is missing the cytoplasmic domain is less effective than wild-type CD4 at enhancing interleukin-2 production by T cells (39). In addition, studies with CD8α', a form of CD8 lacking most of the cytoplasmic tail, have demonstrated that the cytoplasmic domain of CD8 is also required for antigen-induced T-cell activation (50). The cytoplasmic domains of CD4 and CD8 are therefore likely to be involved in positive signaling during T-cell activation. Since it is the cytoplasmic tails of CD4 and CD8 with which p56^{lck} interacts (38, 50), the inability of these truncated forms of CD4 and CD8 to enhance T-cell activation by antigen suggests that p56^{lck} is involved in positive signaling during T-cell activation.

Evidence for negative signaling through CD4, however, comes from the observation that treatment of cells with anti-CD4 antibody, such as GK1.5, blocks Ca^{2+} influx at an early stage of T-cell activation (33, 40) and inhibits both antigen-stimulated and lectin-induced interleukin-2 production (2, 33, 48). It is possible, therefore, that p56^{lck} can also play a role in negative signaling under some circumstances.

Cross-linking of CD4 with antibody is reported to induce tyrosine phosphorylation of p56^{lck} and other cellular proteins, including the ζ subunit of the CD3 complex (42). Cross-linking of CD8 is also reported to have a similar effect in murine thymocytes (46). The physiological significance of the increased phosphorylation of p56^{lck} induced by cross-linking is a subject of some interest. p56^{lck} has two major sites of tyrosine phosphorylation in vivo: Tyr-505 and Tyr-394. Phosphorylation of Tyr-505 inhibits the protein tyrosine kinase activity of p56^{lck} (1, 27). The phosphorylation of Tyr-394 appears to occur by autophosphorylation (7), and the extent of phosphorylation reflects the activity of p56^{lck} (1, 27). It has been reported that the cross-linking of CD4 on murine C8 cells induces an increase in the protein kinase activity of p56^{lck} when assayed in vitro as well as an increase in the phosphorylation of Tyr-505, the inhibitory site (42, 44). These results are paradoxical. We have therefore asked here how general this phenomenon is.

We have also addressed the question of whether it is the binding of antibody to CD4 that induces increased phosphorylation of p56^{lck} on tyrosine or whether the clustering of CD4 molecules is required for this phenomenon. The study of antibody-mediated cross-linking of T-cell surface molecules is of interest because it has the potential to reveal the function of specific proteins during T-cell activation or maturation. It is quite possible, however, that antibody-mediated cross-linking of CD4 or CD8 molecules does not mimic what occurs during antigen-stimulated T-cell activa-

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tion. To address this question, we compared the effect of antibody-mediated cross-linking of CD4 or CD8 with that of antigen stimulation on the tyrosine phosphorylation of cellular proteins.

Finally, since CD4 and CD8 are required for both the activation of mature T cells and positive and negative selection of immature T cells in the thymus, we wanted to know whether the signal transduction through CD4 or CD8 differed in mature and immature T cells. Immature murine thymocytes and mature splenic T cells were therefore isolated, and the effects of cross-linking of CD4 and CD8 on tyrosine phosphorylation of total cellular proteins were studied.

MATERIALS AND METHODS

Cells. SAKRTLS12.1, a murine leukemic $CD4^+8^-$ T-cell line (15), and AKR1, a murine $CD4^+8^+$ leukemic T-cell line (15), were grown in Dulbecco-Vogt modified Eagle medium (DMEM) supplemented with 10% horse serum. L3, a murine $CD4^-8^+$ cytotoxic T-cell line (10), was grown in DMEM supplemented with 10% fetal bovine serum (FBS), 5×10^{-5} M β-mercaptoethanol, and 5% EL-4 cell-conditioned medium as a source of interleukin-2. D10.G4.1, a conalbumin-specific murine $CD4^+8^-$ helper T-cell line (20), was maintained in DMEM with 10% FBS, 5×10^{-5} M β-mercaptoethanol, and 10% concanavalin A-stimulated rat spleen cell culture medium. GA4, an ovalbumin-specific murine $CD4^-8^+$ cytotoxic T-cell clone (4), was grown in RPMI medium supplemented with 10% FBS, 5×10^{-5} M β-mercaptoethanol, 2 mM glutamine, 10 mM *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES; pH 7.2), and 7.5% concanavalin A-stimulated rat spleen cell culture medium. The GA4 T-cell clone was maintained by weekly stimulation of 2×10^5 to 4×10^5 cells with 5×10^6 irradiated syngeneic spleen cells from C57BL/6 mice and 10^5 irradiated E.G7-OVA (29) cells per 2-ml well of 24-well plates (Costar). LB27.4, a B-cell tumor cell line (19) that expresses I-A^b molecules, was maintained in DMEM with 10% FBS and 5×10^{-5} M β-mercaptoethanol.

Thymocytes and spleen cells were prepared from 4-week-old BALB/c mice (bred at the Salk Institute). Splenic T cells were depleted of B cells by panning with polyclonal goat anti-mouse immunoglobulin antibodies (Organon Teknika).

Preparation of Fab fragment. Hybridoma cells secreting monoclonal antibody (MAb) GK1.5 were cultured in serum-free DMEM supplemented with Nutridoma SP (Boehringer Mannheim Biochemicals). Culture supernatant was collected, and GK1.5 was concentrated by ammonium sulfate precipitation (34). The Fab fragment of GK1.5 was generated by incubation of GK1.5 with 5% (wt/wt, enzyme/substrate) immobilized papain (Sigma Chemical Co.) at 37°C for 18 h and purified on a DEAE-cellulose column (26).

Treatments. For the antibody cross-linking experiments, cells were incubated on ice for 30 min in the presence of an excess amount of rat MAb GK1.5 (anti-CD4) or 53.6.72 (anti-CD8) (kindly provided by Bob Hyman). After the excess antibodies were washed away, cells were suspended in serum-free DMEM at room temperature. Cross-linking was performed at room temperature for various periods of time with rabbit anti-rat immunoglobulin G (RAR) antibodies at a final concentration of 20 μg/ml and stopped by the addition of 2× Nonidet P-40 lysis buffer (35) or 2× sodium dodecyl sulfate (SDS)-gel sample buffer.

Ovalbumin (grade V; Sigma) was cleaved by CNBr as described (29), and this preparation (CNBr-OVA) was used in the antigen stimulation experiments. For the binding and presentation of CNBr-OVA, LB cells were pulsed with CNBr-OVA at 500 μg/ml at 37°C for 2 h. Antigenic stimulation was carried out by mixing GA4 cells with an equal number of LB cells that had been preincubated with antigen.

Immunoprecipitation and Western immunoblotting. Cells were lysed in Nonidet P-40 lysis buffer (35) at a concentration of 10^7/ml at 4°C. For the immunoprecipitation of total $p56^{lck}$, the lysate was clarified by centrifugation in a microcentrifuge at maximum speed for 15 min at 4°C. $p56^{lck}$ was then isolated by immunoprecipitation as previously described (14).

Total cellular proteins were prepared by lysing the cells directly in SDS-gel sample buffer and then were analyzed by Western blotting as described elsewhere (14, 17).

Phosphoamino acid analysis. Immunoprecipitated $p56^{lck}$ was purified by electrophoresis on a 15% SDS-polyacrylamide gel and transferred electrophoretically to Immobilon-P (Millipore Corp.) (17). Phosphoamino acid analysis of $p56^{lck}$ bound to Immobilon-P was performed as described previously (18).

Peptide mapping analysis. Immunoprecipitated $p56^{lck}$ was purified by electrophoresis on a 15% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane (Schleicher & Schuell, Inc.). CNBr digestion of $p56^{lck}$ bound to the nitrocellulose membrane was carried out as described previously (24), and the resulting fragments were resolved on a 24% SDS-polyacrylamide gel, using a Tricine cathode buffer (0.1 M Tris, 0.1 M Tricine [Sigma], 0.1% SDS [pH 8.25]). Tryptic mapping analysis of $p56^{lck}$ bound to nitrocellulose was performed as described previously (24).

In vivo $^{32}P_i$ labeling. Cells were washed once with phosphate-free DMEM and then incubated in the same medium supplemented with 10% phosphate-free FBS in the presence of $^{32}P_i$ (0.5 mCi/ml; ICN) at 37°C for 4 h.

In vitro kinase assay. For kinase assays with enolase as a substrate, immunoprecipitates containing $p56^{lck}$ from 5×10^5 cells were incubated with acid-denatured enolase and 10 μCi of [γ-^{32}P]ATP in 20 μl of kinase buffer at room temperature for 1 to 5 min (14).

For kinase assays with [Val^5]-angiotensin II as a substrate, immunoprecipitates containing $p56^{lck}$ from approximately 10^6 cells were incubated with 2 mM angiotensin II (Sigma) and 5 μCi of [γ-^{32}P]ATP in a total volume of 5 μl of kinase buffer (14) at room temperature. The reaction was terminated by the addition of equal volume of 7.5% trichloroacetic acid, and angiotensin was adsorbed onto phosphocellulose paper (Whatman p81). The pieces of paper were then washed in 0.425% phosphoric acid, and [^{32}P]angiotensin was quantified by scintillation counting as described previously (49).

RESULTS

Cross-linking of CD4 or CD8 induces tyrosine phosphorylation of $p56^{lck}$. We examined the effect of antibody-mediated cross-linking of CD4 and CD8 on protein tyrosine phosphorylation in four murine T-cell lines: AKR1, a $CD4^+8^+$ leukemic cell line; SAKR, a $CD4^+8^-$ leukemic cell line; D10, a $CD4^+8^-$ conalbumin-specific helper T-cell line; and L3, a $CD4^-8^+$ cytotoxic T-cell line. Almost 95 to 100% of $p56^{lck}$ is associated with CD4 in AKR1 and SAKR cells and with CD8 in L3 cells (T. R. Hurley, M.-A. Campbell, and B. M. Sefton, unpublished data). The fraction of $p56^{lck}$ that associates with CD4 has not been determined in D10 cells. Conversely, up to 30% of CD4 is bound to $p56^{lck}$ in SAKR

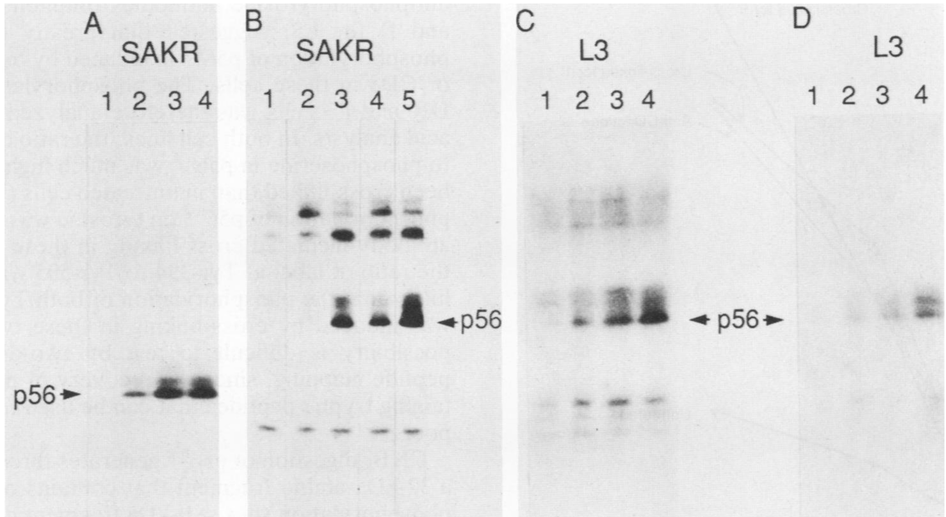

FIG. 1. Induction of tyrosine phosphorylation of p56^{lck} by cross-linking of CD4 or CD8. Total cellular protein was prepared from 4×10^5 T cells before and after cross-linking with GK1.5 (A), Fab fragments of GK1.5 (B), or 53.6.72 (C) plus 20 μg of RAR antibodies per ml as described in Materials and Methods. Immunoprecipitates were prepared from 5×10^5 L3 cells with anti-p56^{lck} antibodies before and after cross-linking of CD8 (D). Tyrosine phosphorylation was analyzed by Western blotting with antiphosphotyrosine antibodies. The fluorograms were exposed at −70°C for 20 h (A), 4 days (C and D) or 2 days (B). (A) Total cellular protein from SAKR cells. Lanes: 1, untreated cells; 2, MAb GK1.5 alone; 3, MAb GK1.5 plus RAR, cross-linked for 1 min; 4, MAb GK1.5 plus RAR, cross-linked for 5 min. (B) Total cellular protein from SAKR cells. Lanes: 1, untreated cells; 2, Fab fragments of GK1.5 alone; 3, GK1.5 alone; 4, Fab fragments of GK1.5 plus RAR, cross-linked for 2 min; 5, GK1.5 plus RAR, cross-linked for 2 min. (C) Total cellular protein from L3 cells. Lanes: 1, untreated cells; 2, MAb 53.6.72 alone; 3, MAb 53.6.72 plus RAR, cross-linked for 1 min; 4, MAb 53.6.72 plus RAR, cross-linked for 5 min. (D) Anti-p56^{lck} immunoprecipitates from L3 cells. Lanes: 1, untreated cells; 2, MAb 53.6.72 alone; 3, MAb 53.6.72 plus RAR, cross-linked for 1 min; 4, MAb 53.6.72 plus RAR, cross-linked for 5 min.

and D10 cells, and 60% of CD8 is bound to p56^{lck} in L3 cells (Hurley et al., unpublished data).

Cells were incubated with a saturating amount of rat MAbs against CD4 (GK1.5) or CD8 (53.6.72) for 30 min on ice and washed, and the antibody was cross-linked by incubation at room temperature with an excess of RAR antibodies. Total cellular protein was then prepared, and tyrosine phosphorylation was analyzed by Western blotting with polyclonal, affinity-purified, antiphosphotyrosine antibodies. Cross-linking of CD4 induced a rapid increase in the tyrosine phosphorylation of a 56-kilodalton (kDa) protein in all three CD4$^+$ T-cell lines: SAKR (Fig. 1A), AKR1 (data not shown), and D10 (data not shown). Cross-linking of CD8 on the L3 (Fig. 1C) and GA4 (see Fig. 5) cytotoxic T-cell clones also stimulated greatly increased tyrosine phosphorylation of a 56-kDa protein. Western blotting with antiphosphotyrosine antibodies of immunoprecipitates prepared from these cells with anti-p56^{lck} antibodies showed that the phosphorylation of p56^{lck} on tyrosine was stimulated by cross-linking and suggested that the 56-kDa band seen in the total cellular lysates was p56^{lck} (Fig. 1D; see below). The induction was maximal within 1 min of cross-linking (Fig. 1A, C, and D, lanes 3).

Treatment of cells with anti-CD4 or anti-CD8 alone had a noticeable effect on the tyrosine phosphorylation of p56^{lck} (Fig. 1A, C, and D, lanes 2; Fig. 1B, lane 3). To test whether this increase in tyrosine phosphorylation of p56^{lck} resulted from the binding of the antibody to CD4 or from the dimerization of the CD4 molecules induced by the bivalent anti-CD4, we examined the effect of monovalent Fab fragments of anti-CD4 (Fig. 1B). The ability of this preparation of Fab fragments of GK1.5 to bind to surface CD4 molecules was confirmed by flow cytometry (data not shown). Treatment of SAKR cells with Fab fragments of GK1.5 did not induce any alteration in the tyrosine phosphorylation of p56^{lck} (Fig. 1B, lane 2). Inclusion of RAR antibodies in addition to the Fab fragments of anti-CD4, however, led to a significant increase in tyrosine phosphorylation of p56^{lck} (Fig. 1B, lane 4). This result again showed that this preparation of Fab did in fact bind to CD4 and that the alterations in the tyrosine phosphorylation of cellular proteins shown here depended on the cross-linking of CD4, at least into dimers.

In vitro kinase activity of p56^{lck}. It has been reported that the tyrosine kinase activity of p56^{lck}, when assayed in vitro with enolase as an exogenous substrate, is increased 300 to 500% by cross-linking with anti-CD4 (44). In our hands, however, when enolase was used as an exogenous substrate, cross-linking of CD4 or CD8 induced increases in activity of 0, 70, 130, and 58% in AKR1, SAKR, D10, and L3 cell lines, respectively (Table 1). Disappointed by these results, we looked for changes in the kinase activity of p56^{lck} by using [Val^5]-angiotensin II as an exogenous substrate.

TABLE 1. In vitro kinase assays of p56^{lck}

Cell line	Fold stimulation of the kinase activity of p56^{lck} by cross-linking[a]	
	Enolase	[Val^5]-angiotensin II
AKR1	1.0	3.5
SAKRTLS12.1	1.7	4.0
D10.G4.1	2.3	2.5
L3	1.6	1.6

[a] The kinase activity of p56^{lck} isolated by immunoprecipitation with anti-p56^{lck} antibodies was assayed as described in Materials and Methods. Under all circumstances, incorporation of ^{32}P in the indicated exogenous substrate was linear with time for more than 5 min. The activity of p56^{lck} was calculated from the rate of incorporation of ^{32}P.

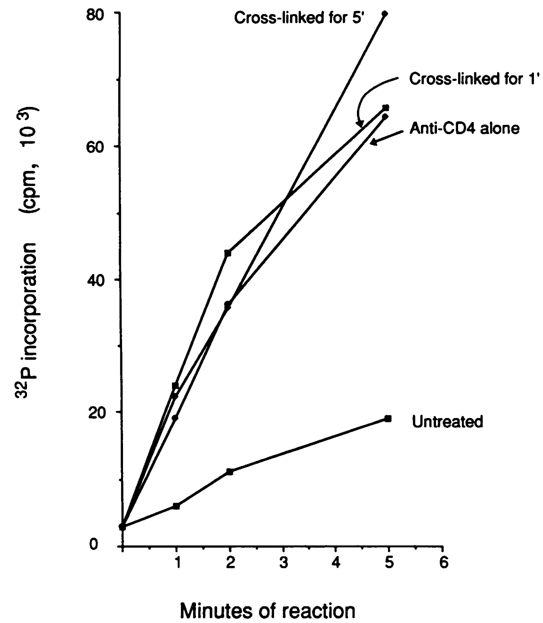

FIG. 2. Stimulation of in vitro kinase activity of p56^{lck} by cross-linking of CD4 on SAKR cells. Immunoprecipitates were prepared with anti-p56^{lck} antibodies from 10^6 SAKR cells before and after cross-linking with GK1.5 plus RAR as described in Materials and Methods. In vitro kinase assays were conducted with 2 mM [Val^5]-angiotensin II peptide as a substrate as described in Materials and Methods. The graph shows incorporation of ^{32}P into angiotensin as a function of kinase reaction time.

When assayed this way, cross-linking of CD4 with either anti-CD4 alone or anti-CD4 plus rabbit anti-rat antibodies induced an approximately fourfold increase in kinase activity of p56^{lck} in AKR1 and SAKR cells (Table 1 and Fig. 2) and a two- to threefold increase in D10 cells (Table 1). In contrast, however, little alteration in in vitro kinase activity could be detected after cross-linking of CD8 on L3 cells (Table 1).

Determination of the sites in p56^{lck} whose phosphorylation is induced by antibody cross-linking. We determined the sites in p56^{lck} whose phosphorylation was induced during cross-linking by two-dimensional tryptic peptide mapping. p56^{lck} was isolated by immunoprecipitation with anti-p56^{lck} antibodies from cells that had been labeled biosynthetically with ^{32}P$_i$ and then cross-linked with either anti-CD4 or anti-CD8. Two major phosphotyrosine-containing peptides, peptide 1 and peptide 2, that contain Tyr-394 and Tyr-505, respectively (7, 27), were seen in maps of p56^{lck} from untreated cells (Fig. 3a, c, e, and g). Peptide 3 is derived from the amino-terminal half of the molecule and contains phosphoserine (25, 45).

Cross-linking of CD4 induced a rapid and obvious increase in the phosphorylation of Tyr-394 (peptide 1) in p56^{lck} from AKR1 cells (Fig. 3a and b) and SAKR cells (Fig. 3c and d). Phosphorylation of Tyr-505 (peptide 2) was also increased relative to that of the phosphoserine-containing peptide 3 in p56^{lck} from SAKR cells (Fig. 3c and d) but to a lesser extent than that of Tyr-394.

In contrast, the ratio of phosphate label in the peptides containing Tyr-394 and Tyr-505 changed only a little after cross-linking of CD4 on D10 cells (Fig. 3e and f) and did not change significantly after cross-linking of CD8 on L3 cells (Fig. 3g and h). This result was surprising, since blotting with antiphosphotyrosine antibodies (unpublished data; Fig. 1C and D for L3) suggested that greatly increased tyrosine phosphorylation of p56^{lck} is induced by cross-linking of CD4 or CD8 in these cells. The phosphorylation of p56^{lck} from D10 and L3 cells was therefore analyzed by phosphoamino acid analysis. In both cell lines, the ratio of phosphotyrosine to phosphoserine in p56^{lck} was much higher in cells that had been cross-linked than in untreated cells (Fig. 4A). Since the phosphorylation of p56^{lck} on tyrosine was clearly induced by antibody-mediated cross-linking in these two cell lines, but the ratio of label at Tyr-394 to Tyr-505 was unchanged, it is likely that the phosphorylation of both Tyr-394 and Tyr-505 was induced by cross-linking in these two cell lines. This possibility is difficult to test by two-dimensional tryptic peptide mapping, since the recovery of phosphoserine-containing tryptic peptides that can be used for normalization is poor.

CNBr digestion of p56^{lck} generates three major fragments: a 32-kDa amino fragment that contains one or more serine phosphorylation sites, a 9-kDa fragment containing Tyr-394, and a 4-kDa fragment containing Tyr-505 (25, 45). This technique can therefore also be used to study the phosphorylation of p56^{lck}. As expected, cross-linking of CD4 in SAKR cells labeled biosynthetically with ^{32}P$_i$ increased the tyrosine phosphorylation of the 9-kDa fragment dramatically and the phosphorylation of the 4-kDa fragment moderately but had little effect on the phosphorylation of the amino terminal 32-kDa fragment (Fig. 4B). This finding agrees with the result derived from tryptic peptide mapping.

In D10 cells, however, the labeling of both the 9- and 4-kDa fragments, relative to that of the 32-kDa fragment, increased after cross-linking (Fig. 4B). This result suggests that phosphorylation of both Tyr-394 and Tyr-505 was stimulated and to approximately the same extent. This finding is consistent with the results of the two-dimensional tryptic peptide mapping. Similarly, in L3 cells, the labeling of both the 9- and 4-kDa fragments, but not the 32-kDa fragment, was induced by cross-linking (data not shown). This too is consistent with the results obtained by tryptic peptide mapping.

All of the mapping experiments described above analyzed the total population of p56^{lck} isolated by immunoprecipitation with antibodies against p56^{lck}. Similar results were obtained with the population of p56^{lck} bound to CD4 (data not shown).

Comparison between the effect of antigenic stimulation and cross-linking of CD8 on tyrosine protein phosphorylation in GA4 cells. We addressed the question of whether the cross-linking of CD4 or CD8 mimics what occurs during antigen-induced T-cell activation by comparing the tyrosine phosphorylation of total cellular proteins during antigen-induced T-cell activation and antibody cross-linking. Total cellular proteins from GA4 cells that had been either cross-linked with anti-CD8 plus RAR antibodies or stimulated with CNBr-OVA on antigen-presenting cells were analyzed by Western blotting with antiphosphotyrosine antibodies (Fig. 5). As expected, cross-linking of CD8 induced a rapid increase in the tyrosine phosphorylation of p56^{lck} but had little effect on the tyrosine phosphorylation of other cellular proteins. In contrast, antigenic stimulation of GA4 cells induced the tyrosine phosphorylation of 120-, 105-, 90-, 60-, and 40-kDa proteins rapidly but had little effect on the phosphorylation of p56^{lck} (Fig. 5). Similar results were obtained with three other antigen-dependent murine T-cell clones (data not shown).

Comparison of the effect of cross-linking of CD4 and CD8 on

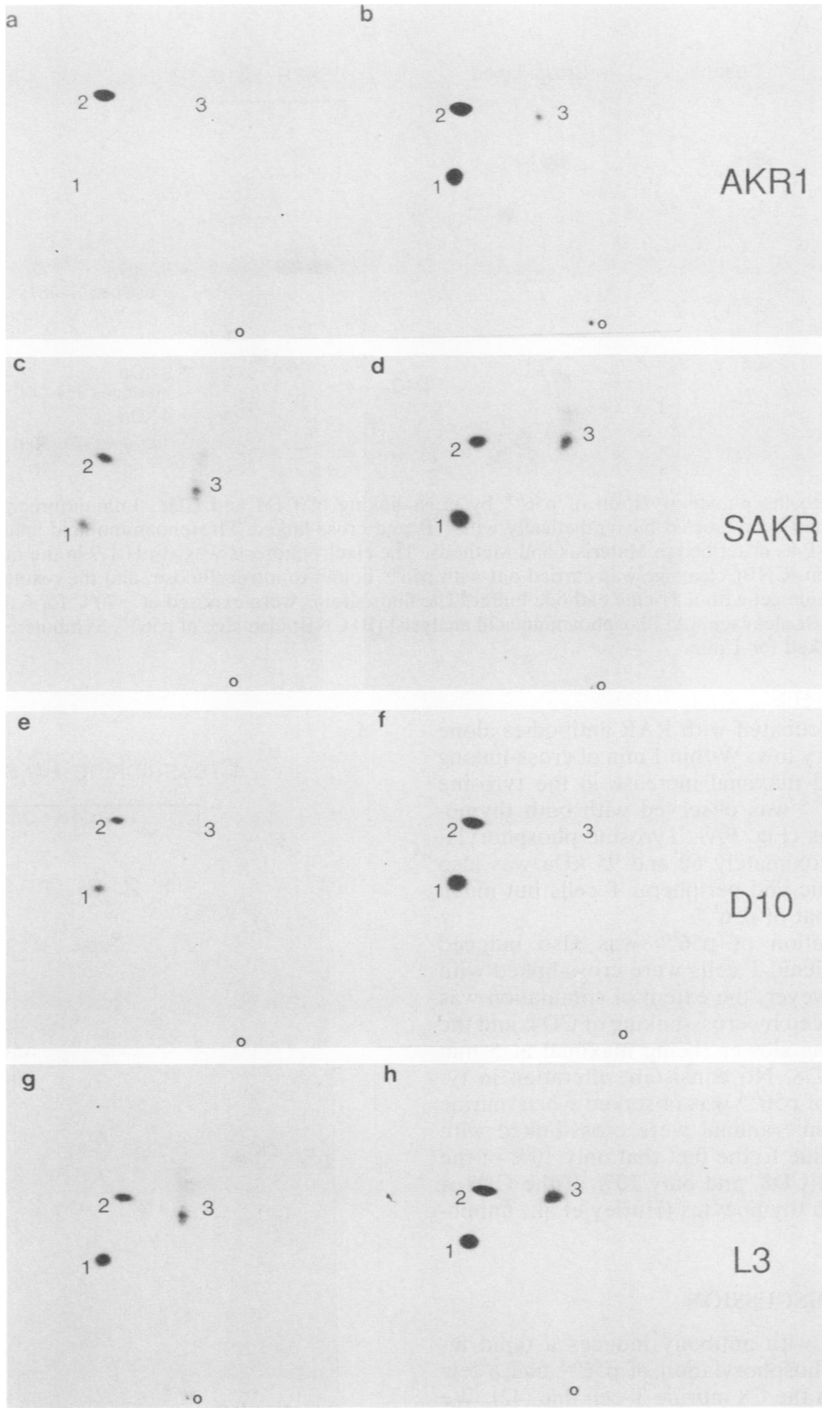

FIG. 3. Two-dimensional tryptic peptide mapping of p56^{lck}. Immunoprecipitates were prepared with anti-p56^{lck} from cells that had been labeled biosynthetically with $^{32}P_i$ and cross-linked as described in Materials and Methods. p56^{lck} bound to nitrocellulose was digested with trypsin, and the resulting peptides were analyzed by electrophoresis at pH 8.9 in the horizontal dimension and ascending chromatography in the vertical dimension. The fluorograms were exposed at −70°C for 3 days (a and b) or 5 days (c to h). (a, c, e, and g) Untreated cells; (b, d, and f) MAb GK1.5 plus RAR, cross-linked for 1 min; (h) MAb 53.6.72 plus RAR, cross-linked for 1 min.

murine thymocytes with that on splenic T cells. It has been shown that in murine splenic T cells and thymocytes, at least 35 to 50% of the p56^{lck} is associated with CD4 and 10% is associated with CD8. Conversely, 30% of CD4 and 10% of CD8 is bound to p56^{lck} in thymocytes, and 40% of CD8 is bound to p56^{lck} in splenocytes (Hurley et al., unpublished data). The effect of antibody-mediated cross-linking of CD4 or CD8 on tyrosine phosphorylation in thymocytes and B-cell-depleted spleen cells prepared from 4-week-old BALB/c mice was examined by Western blotting with antiphosphotyrosine antibodies. The constitutive level of tyrosine phosphorylation in untreated cells (Fig. 6, lanes 1) or

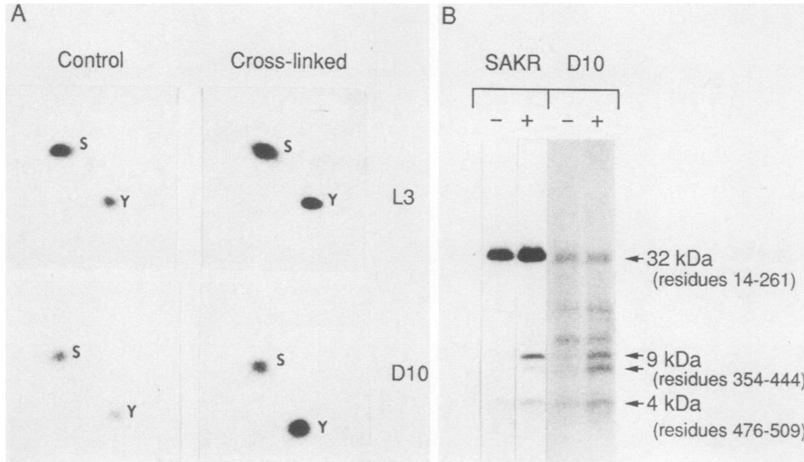

FIG. 4. Induction of tyrosine phosphorylation of $p56^{lck}$ by cross-linking of CD4 and CD8. Immunoprecipitates were prepared with anti-$p56^{lck}$ from cells that had been labeled biosynthetically with $^{32}P_i$ and cross-linked. Phosphoamino acid analyses were carried out with $p56^{lck}$ bound to Immobilon-P as described in Materials and Methods. The electrophoresis was at pH 1.9 in the horizontal dimension and pH 3.5 in the vertical dimension. CNBr cleavage was carried out with $p56^{lck}$ bound to nitrocellulose, and the resulting peptides were analyzed on a 24% SDS-polyacrylamide gel with a Tricine cathode buffer. The fluorograms were exposed at −70°C for 6 days for phosphoamino acid analyses and 2 days for CNBr cleavage. (A) Phosphoamino acid analysis; (B) CNBr cleavage of $p56^{lck}$. Symbols: −, Untreated cells; +, MAb GK1.5 plus RAR, cross-linked for 1 min.

in cells that had been incubated with RAR antibodies alone (data not shown) was very low. Within 1 min of cross-linking of CD4, a dramatic and maximal increase in the tyrosine phosphorylation of $p56^{lck}$ was observed with both thymocytes and splenic T cells (Fig. 6A). Tyrosine phosphorylation of proteins of approximately 60 and 95 kDa was also stimulated in both thymic and peripheral T cells but much less dramatically than that of $p56^{lck}$.

Tyrosine phosphorylation of $p56^{lck}$ was also induced slightly when murine splenic T cells were cross-linked with anti-CD8 (Fig. 6B). However, the extent of stimulation was much less than that induced by cross-linking of CD4, and the kinetics of induction was slower, being maximal at 5 min after cross-linking of CD8. No consistent alteration in tyrosine phosphorylation of $p56^{lck}$ was observed when murine thymocytes from the same animal were cross-linked with anti-CD8. This may be due to the fact that only 10% of the $p56^{lck}$ is associated with CD8, and only 10% of the CD8 is associated with $p56^{lck}$, in thymocytes (Hurley et al., unpublished).

DISCUSSION

Cross-linking of CD4 with antibody induces a rapid increase in the tyrosine phosphorylation of $p56^{lck}$ and a few other cellular proteins in the C8 murine T-cell line (42). We have observed a similar effect after cross-linking of CD4 with antibody in murine thymocytes, splenic T cells, and the AKR1, SAKR, and D10 cell lines. Cross-linking of CD8 also stimulated tyrosine phosphorylation of $p56^{lck}$ dramatically in L3 and GA4 cells and slightly in splenic T cells. We observed little or no induced tyrosine phosphorylation in thymocytes and AKR1 cells after cross-linking with anti-CD8. Our results differ from those reported previously (46) that cross-linking of CD8 on murine thymocytes induces tyrosine phosphorylation of $p56^{lck}$. The lack of an obvious alteration in the phosphorylation of $p56^{lck}$ in thymocytes and AKR1 cells in response to cross-linking of CD8 might reflect the fact that no more than 25% of $p56^{lck}$ is associated with CD8

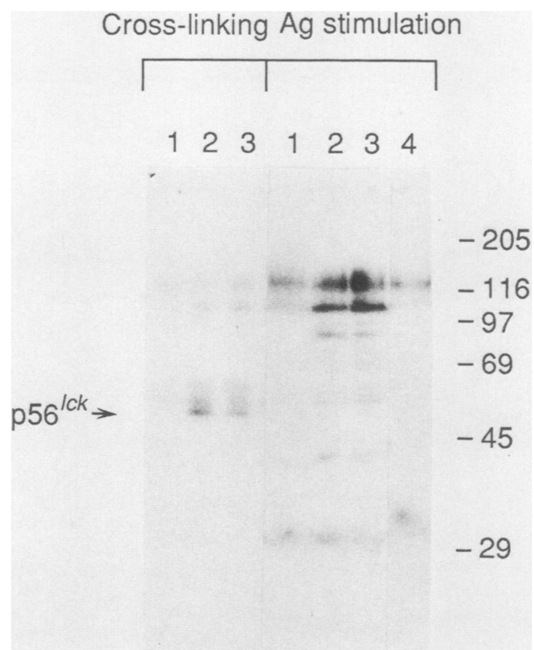

FIG. 5. Western blotting of total cellular protein from GA4 cells with antiphosphotyrosine antibodies. Cells were either cross-linked with anti-CD8 plus RAR or stimulated with an equal number of LB cells that had been preincubated with CNBr-OVA. Total cellular protein was prepared from 4×10^5 GA4 cells and fractionated on a 15% SDS-polyacrylamide gel. Western blotting was carried out as described in Materials and Methods. The fluorogram was exposed for 4 days at −70°C. (Cross-linking panel) Lanes: 1, untreated cells; 2, MAb 53.6.72 plus RAR, cross-linked for 1 min; 3, MAb 53.6.72 plus RAR, cross-linked for 5 min. (Antigen [Ag] stimulation panel) Lanes: 1, GA4 plus LB cells; 2, GA4 plus LB cells preincubated with CNBr-OVA, stimulated for 1 min; 3, GA4 plus LB cells preincubated with CNBr-OVA, stimulated for 5 min; 4, LB cells preincubated with CNBr-OVA only.

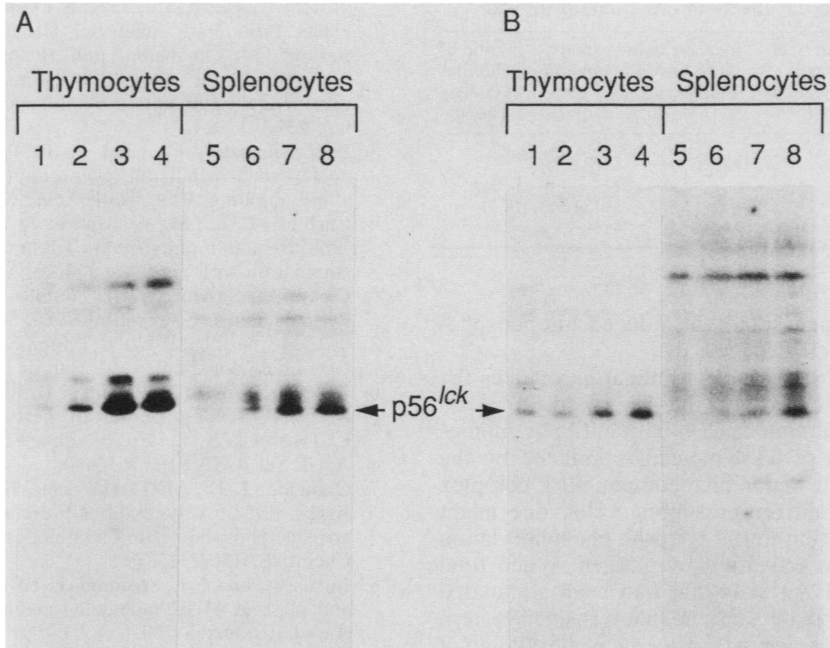

FIG. 6. Effect of cross-linking of CD4 and CD8 on murine thymocytes and splenic T cells. Thymocytes and splenic T cells prepared from BALB/c mice were cross-linked with GK1.5 or 53.6.72 plus RAR as described in Materials and Methods. Total cellular protein from 2×10^6 cells was analyzed by Western blotting with antiphosphotyrosine antibodies. The fluorograms were exposed at $-70°C$ for 20 h for thymocytes and 3 days for splenocytes. (A) Cross-linking of CD4 on thymocytes and splenic T cells. Lanes: 1, untreated cells; 2, MAb GK1.5 alone; 3, MAb GK1.5 plus RAR, cross-linked for 1 min; 4, MAb GK1.5 plus RAR, cross-linked for 5 min; 5, untreated cells; 6, MAb GK1.5 alone; 7, MAb GK1.5 plus RAR, cross-linked for 1 min; 8, MAb GK1.5 plus RAR, cross-linked for 5 min. (B) Cross-linking of CD8 on thymocytes and splenic T cells. Lanes: 1, untreated cells; 2, MAb 53.6.72 alone; 3, MAb 53.6.72 plus RAR, cross-linked for 1 min; 4, MAb 53.6.72 plus RAR, cross-linked for 5 min; 5, untreated cells; 6, MAb 53.6.72 alone; 7, MAb 53.6.72 plus RAR, cross-linked for 1 min; 8, MAb 53.6.72 plus RAR, cross-linked for 5 min.

in these cells (Hurley et al., unpublished). Whether the differing effects of cross-linking of CD8 in thymocytes and splenocytes on the phosphorylation of p56^{lck} reveal some important difference between CD8-mediated signaling in immature and mature T cells is not yet clear. The effects of cross-linking of CD4 on the tyrosine phosphorylation of p56^{lck} appeared to be similar in immature and mature T cells.

It has been reported that cross-linking of CD4 on C8 cells induces a three- to fivefold increase in the kinase activity of p56^{lck} when assayed in vitro with enolase as an exogenous substrate (44). Our efforts to detect such an increase in kinase activity of p56^{lck} in the four cell lines with enolase as an exogenous substrate yielded unsatisfying results (Table 1). However, when we used [Val^5]-angiotensin II as a substrate in the in vitro kinase assay, we could consistently detect a four- to fivefold increase in the kinase activity of p56^{lck} in the SAKR and AKR1 cell lines after cross-linking of CD4. Angiotensin may therefore be a more sensitive exogenous substrate of p56^{lck} than enolase. Despite the fact that the tyrosine phosphorylation of p56^{lck} was induced in all four cell lines, the in vitro kinase activity of p56^{lck} was increased significantly by cross-linking in only three of the four cell lines.

It is possible that cross-linking of CD4 or CD8 has different effects on the in vitro kinase activity of p56^{lck} in different cells because it has different effects on the phosphorylation of p56^{lck}. In all four cell lines, antibody-mediated cross-linking stimulated the phosphorylation of Tyr-394, the autophosphorylation site. In D10 and L3 cells, however, the phosphorylation of Tyr-505 was also stimulated and to a similar extent to that of Tyr-394. Why cross-linking affects the phosphorylation of p56^{lck} differently in these cell lines is not clear. It could be that the ability of cross-linking to induce the inhibitory phosphorylation of p56^{lck} on Tyr-505 is lost in leukemic cell lines. Veillette et al. observed an increase in the phosphorylation of only Tyr-505 after cross-linking of CD4 in C8 cells (42). We did not observe this in any cell lines that we studied.

The ability of anti-CD4 or anti-CD8 antibodies to stimulate the tyrosine phosphorylation of p56^{lck} could result either from the simple binding of antibody to CD4 or CD8 or from the oligomerization of CD4 or CD8 molecules induced by multivalent antibodies. Oligomerization of CD4 molecules is apparently essential, since Fab fragments of anti-CD4 are inactive in inducing tyrosine phosphorylation unless they are cross-linked with a second antibody. Dimerization appears to be sufficient since anti-CD4 or anti-CD8 induces an increase in tyrosine phosphorylation of p56^{lck} in the absence of a second, cross-linking antibody.

This requirement of dimerization for autophosphorylation and activation of p56^{lck} is similar to what has been found with the growth factor receptor tyrosine kinases such as the epidermal growth factor receptor, platelet-derived growth factor receptor, and colony-stimulating factor 1 receptor. In each case, ligand-induced receptor oligomerization is necessary for induced autophosphorylation, which in turn is required for activation of the tyrosine kinase activity of the receptor (3, 12, 13, 36). How dimerization stimulates the kinase activity and the phosphorylation of p56^{lck} is not yet clear. The increased autophosphorylation of p56^{lck} could result from either intermolecular phosphorylation or intramolecular phosphorylation, although the requirement for the

TABLE 2. Summary of the effects of cross-linking on p56^{lck}

Cell line	Increase in total tyrosine phosphorylation of p56^{lck}	Increase in in vitro kinase activity of p56^{lck}	Increase in ratio of phosphorylation of p56^{lck} at Tyr-394 to that at Tyr-505
AKR1	+	+	+
SAKRTLS12.1	+	+	+
D10.G4.1	+	−	−
L3	+	−	−

dimerization seems to suggest that the induced autophosphorylation is an intermolecular event.

It has been reported that antigen stimulation induces the clustering of CD4 molecules with the T-cell receptor–CD3 complex (23). If cross-linking of CD4 with antibody mimics in part the aggregation of CD4 molecules induced by the interaction of CD4 with major histocompatibility complex class II molecules on antigen-presenting cells, one might expect to find an alteration in the tyrosine phosphorylation of p56^{lck} to accompany activation by antigen. When total cellular proteins from GA4 cells that had been stimulated with antigen were analyzed by antiphosphotyrosine Western blotting, no dramatic increase in tyrosine phosphorylation of p56^{lck} was observed.

It is possible, therefore, that the signal generated by cross-linking of CD4 or CD8 with antibody differs quantitatively or qualitatively from that generated by antigenic stimulation. It could be that the effect of cross-linking with antibody is a very exaggerated, but otherwise faithful, reflection of what happens during normal T-cell activation. Alternatively, it is possible that the effects of cross-linking are misleading and that the phosphorylation or activity of p56^{lck} is in fact unchanged during T-cell activation. Even if this were the case, the phosphorylation of substrates by p56^{lck} could still play a role in T-cell activation. T-cell activation might affect the interaction of p56^{lck} with specific polypeptide substrates, perhaps as a result of the induced clustering of CD4 with the T-cell receptor, and this could lead to the increased phosphorylation of polypeptide substrates on tyrosine without affecting the intrinsic activity of p56^{lck}.

If the phosphorylation of polypeptide substrates by p56^{lck} is involved in signaling during T-cell maturation and T-cell activation, an important question is whether p56^{lck} generates a positive or a negative signal. Depending on how the assay is done, CD4 and CD8 can be shown to deliver both positive and negative signals in cultured cells (2, 5, 16, 33, 39, 48, 50). However, because the cytoplasmic domains of both CD4 and CD8, with which p56^{lck} interacts, have been shown to be important in the activation of several T-cell lines by antigen (39, 50), it appears likely that p56^{lck} can participate in positive signaling under some circumstances.

ACKNOWLEDGMENTS

We are grateful to R. Hyman, I. Engle, J. Kaye, S. Hedrick, F. Carbone, and A. Glasebrook, who generously provided antibodies, cells, and advice. We also thank H. Ostergaard and I. Trowbridge for helpful discussions and T. Hurley, H. Adler, M.-A. Campbell, T. Hunter, and P. Reynolds for critically reading the manuscript.

This work was supported by Public Health Service grants CA 14195 and CA 42350 from the National Cancer Institute.

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